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PRODUCTION OF STORAGE LIPIDS FROM NITROGEN-RICH SUBSTRATE

Master of Science Thesis

Examiners: Assist. prof. Ville Santala
and PhD Suvi Santala
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Acinetobacter baylyi ADP1 on bakteeri, joka pystyy tuottamaan vahaestereitä typpiköyhissä olosuhteissa. Vahaesterit ovat rasvahappojen ja pitkäketjuisten alkoholien estereitä ja niitä käytetään muun muassa kosmetiikassa. Monet teollisuuden alat tuottavat typpirikasta jätettä, joiden hyötykäyttö olisi tärkeä tavoite.

Tämän dilpomityön tavoite oli tutkia voidaanko ADP1 muokata geneettisesti niin, että se tuottaisi vahaestereitä normaalia tehokkaamin typpirikkaissa olosuhteissa. Tätä varten rakennettiin plasmidi, joka sisältää arabinoosilla indusoitavan promoottorin ja geenin "solun tukirankaan sitoutuvalle myrkylle" (cbtA). *Escherichia colista* saadun cbtA:n sisältävä konstrukti integroitiin tämän jälkeen ADP1:n genomiin. Cbta pysäyttää solujen jakautumisen, mikä voisi johtaa suurempaan vahaesterien tuottoon. Muita solujakautumisen pysäytyskeinoja testattiin myös hypoteesin tutkimiseen. Solujen jakautuminen pysäytettiin yhdessä testissä antibiooteilla ja toisessa vaihtamalla hiililähdettä.

Arabinoosin lisäys ei vaikuttanut transformoituihin soluihin, vaan niiden kasvunopeus oli yhtä suuri kuin kontrollisoluilla. Cbta:n vaikutusta vahaesterien tuottoon typpirikkaissa olosuhteissa ei tämän vuoksi voitu arvioida. Kaksi muuta testiä, joissa solujakautuminen pysäytettiin, osoittivat, että jakautumisen pysäyttäminen voi johtaa suurempiin vahaesterituottoihin typpirikkaissa olosuhteissa.

ABSTRACT

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Acinetobacter baylyi ADP1 is a bacterium capable of producing wax esters (WE) in nitrogen limited conditions. WEs are esters of fatty acids and fatty alcohols and they are used in e.g. cosmetics. Many fields of industry produce nitrogen-rich wastes. The utilization of these wastes would be a good objective.

The purpose of the thesis was to study whether ADP1 can genetically modified to produce WEs more efficiently in nitrogen-rich conditions. In order to achieve more efficient production, a plasmid with a gene for a cytoskeleton-binding toxin (cbtA) from *Escherchia coli* and with an arabinose inducible promoter was constructed and then transformed into ADP1 cells. CbtA stops cell growth, which could yield in a better WE accumulation. Other cell growth stopping methods were used as well to test this hypothesis. The cell growth was stopped in two different tests with antibiotics and by changing the carbon source.

Arabinose induction did not affect the transformed cells, as their cell growth rate remain unchanged compared to the controls. Thus the effect of cbtA on WE accumulation on n-rich conditions could not be evaluated. However, additional tests showed that stopping the cell growth could yield in better WE levels in n-rich conditions.

PREFACE

The research for this thesis was conducted in the facilities of the Laboratory of Chemistry and Bioengineering in Tampere University of Technology. Bacterial strains constructed in the laboratory in earlier studies were used in this work.

I want to thank the examiners of this thesis, assistant professor Ville Santala and PhD Suvi Santala. Other members of the laboratory deserve a thank you as well, especially doctoral student Tapio Lehtinen. The support of my family and friends was extremely valuable during this long thesis process.

Helsinki 21.05.2018

Niko Kalinainen

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LIST OF ABBREVIATIONS

ACC1	Acetyl-CoA carboxylase
aceA	A isocitrate lyase gene
ADP1	*Acinetobacter baylyi*
AGAT	Acyl-G3P acyltransferase
AGPase	ADP-glucose pyrophosphorylase
AI	Autoinducer
acr1	A fatty-acyl-Coenzyme A reductase gene
BnaDGAT	*Brassica napus*diacylglycerol acyltransferase
CaMV	Cauliflower mosaic virus
cbtA	Cytoskeleton binding toxin A
CIS	Citrate synthase
CoA	Coenzyme A
DGAT	Diacylglycerol acyltransferases
G3P	Glycerol-3-phosphate
G3PDH	Glycerol-3-phosphate dehydrogenase
GPAT	Glycerol-3-phosphate acyltransferase
Gpd1	A glycerol-3-phosphate dehydrogenase gene
GUT1	Glycerol kinase
HPLC	High partition liquid chromatography
icd	A isocitrate dehydrogenase gene
idh	A isocitrate dehydrogenase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Lysogen broth
LPA	Lysophosphatidic acid
LPAAT	Lysophosphatidic acid acyltransferase
OD	Optical density
PA	Phosphatidic acid
PAP	Phosphatidic acid phosphatase
PCR	Polymerase chain reaction
PRAI	Phosphoribosylanthranilate isomerase
RbcS	Ribulose-1,5-bisphosphate carboxylase small subunit
SCD	Stearoyl-CoA desaturase
TAG	Triacylglycerol

TLC	Thin layer chromatography
UV	Ultra violet
WE	Wax ester
WS/DGAT	Wax ester synthase/acyl-CoA:diacylglycerol acyltransferase

1. INTRODUCTION

Acinetobacter baylyi ADP1, a Gram-negative bacterium, is capable of producing wax esters and triacylglycerols in carbon rich and nitrogen limited conditions (Fixter et al. 1986). ADP1 is a great model organism since it is not pathogenic and it has many same properties as vastly studied *Escherichia coli* (Barbe et al. 2004; Metzgar et al. 2004). In addition, it is naturally transformable (Juni and Janik 1969).

Wax esters (WE) are formed from fatty acids and fatty alcholols i.e. long-chained acids and alcohols (Holum 1990; Schmid 1995). They are used e.g. pharmaceuticals and cosmetics. WEs can be found in nature in plants and fish and other bacteria than ADP1 can produce them as well.(Schmid 1995) Although, ADP1 can produce WEs in nitrogen limited conditions, it struggles to produce them in nitrogen-rich. One possible reason for this might be that the cells use WEs as energy and carbon reserve molecules.(Fixter et al. 1986)

By stopping the cell division of the ADP1 cells, it might be possible to increase the WE yield in nitrogen-rich conditions. The purpose of this thesis was to study whether ADP1 can produce more WEs from nitrogen-rich substrate when its cell division is disrupted. This is done by using metabolic engineering to transform a cytoskeleton-binding toxin producing gene from *E. coli*. The most feasible chassis for the transformation is chosen by growing different ADP1 strains with nitrogen-rich substrate. Additional experiments including growth stopping by changing the carbon source and with chloramphenicol are conducted to explore the hypothesis.

2. BACKGROUND

In this chapter, the theoretical background important for understanding the conducted studies is presented. This includes the basic principles of metabolic engineering, knowledge about *Acinetobacter baylyi* and information about cytoskeleton-binding toxin cbtA, which is used in this study. In addition, earlier studies focusing on storage lipid production by microbes are introduced.

2.1 Metabolic Engineering

In the first review written about metabolic engineering, James E. Bailey describes it as "the improvement of cellular activities by manipulation of enzymatic, transport and regulatory functions of the cell with the use of recombinant DNA technology." (Bailey 1991). Recombinant DNA technology consist of inserting the target DNA into a cloning vector, transferring the vector into the host cell, selecting the transformed cells and finally expressing the target gene in the host cell (Glick et al. 2010). Figure 1 illustrates the procedure commonly applied in recombinant DNA technology.

Nowadays a more popular method, where the target gene is integrated directly into the host organisms genome, is used. Often a genome integration cassette construct with a homologous sequence with the host genome is created. The construct is then transformed into electrocompetent cells via electroporation.(Sharan et al. 2009)

To choose the target for metabolic engineering, different aspects of the organisms metabolism have to be considered. The physiology has to be studied in detail, metabolic flux and the control of the metabolic routes have to be analysed, the thermodynamics of the pathways have to be understood and kinetic modelling has to be done.(Nielsen 1997)

Metabolic flux analysis is used to determine the flow of carbon in the metabolic

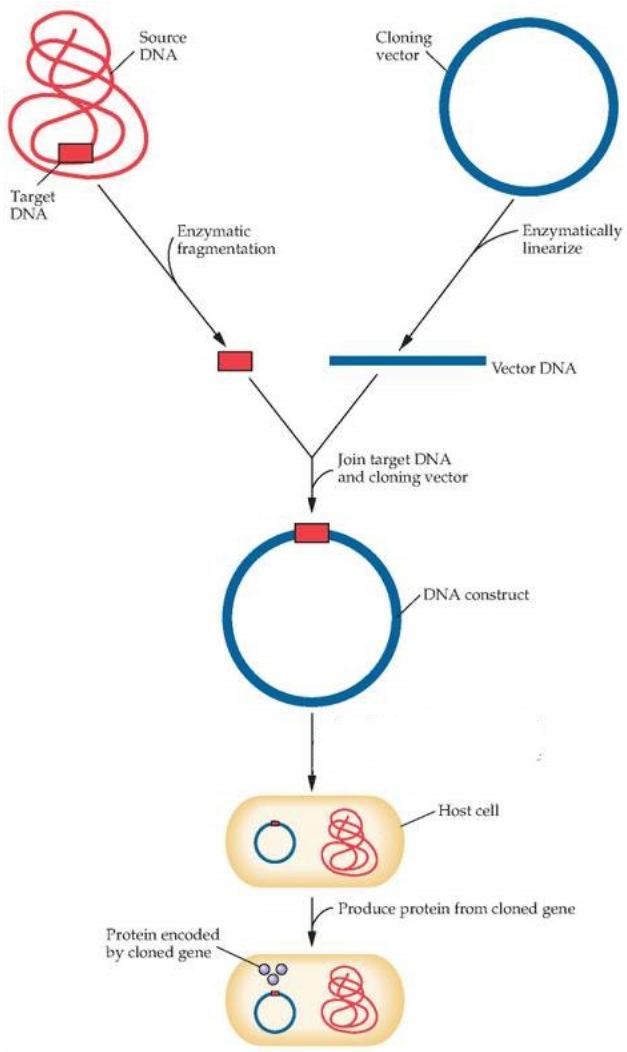


Figure 1. *Using recombinant DNA technology to express proteins coded by a desired target gene.(Adapted from Glick et al. 2010)*

network of interest (Varma and Palsson 1994). Earlier the analysis was done with information about reaction stoichiometry for the metabolic reactions, growth requirements for the organism and other parameters (Vallino and Stephanopoulos 1990). Nowadays, ^{13}Carbon is used in metabolic flux analyses to determine the distribution of single carbons in the central metabolism of an organism. A substrate consisting of ^{13}C is fed to the organism and the carbon is used by it to produce different metabolites. The ^{13}C can be then recognized from the end products by nuclear magnetic resonance measurements.(Marx et al. 1996)

The metabolic flux analysis gives information only about the distribution between

different branch points. Metabolic control analysis is needed to assess how the metabolite production is kept in balance.(Nielsen 1997) This can be achieved by calculating elasticity coefficients and control coefficients for the metabolic network (Fell and Sauro 1985).

The feasibility of the pathway is studied by calculating equilibrium constants or Gibbs free energies for the reactions. The concentration range, in which the pathway is feasible, can be calculated and the reactions compromising the feasibility can be identified.(Mavrovouniotis 1993)

In these analyses, steady state is assumed, but it is important to understand the metabolic systems further. If the metabolite concentrations change or more substrate is added, steady state calculations do not apply. Thus mathematical models are used to analyse metabolic pathways further.(Gombert and Nielsen 2000) E.g. the group of Rizzi was among the first to implement a mathematical model to describe the glycolysis of *Saccharomyces cerevisiae* in 1997 (Rizzi et al. 1997).

After the metabolism has been analysed, it could be seen how the desired metabolite yield could be increased. One approach is to extend the substrate range of an organism (Cameron and Tong 1993). For example, micro-organisms have been engineered to utilize cellulose as a carbon source (Cho et al. 1999; Ko et al. 2016).

Another form of metabolic engineering is to engineer an organism to produce new products. This can be done by expressing heterologous proteins to extend a pathway or introducing completely new pathways to the organism. Often the desired metabolite yield is increased by eliminating or reducing the formation of other compounds produced by the pathway. Another approach is to engineer the physiology of the organism. This might be done to improve its adaptation to different conditions or to change the morphology cell to support better metabolite yield.(Nielsen 2001)

Increasing the activity of a certain biochemical reaction can increase the yield of a metabolite. This can be done by overexpressing the enzyme related to the reaction. The overexpression can be achieved e.g. by using an inducible promoter.(Nielsen 2001)

2.2 *Acinetobacter baylyi*, ADP1

Acinetobacter baylyi (ADP1) is a Gram-negative bacterium found in soil and it possesses the ability to produce storage lipids i.e. triacylglycerols (TAG) and wax esters, especially in nitrogen limited conditions (Carr et al. 2003; Fixter et al. 1986; Kalscheuer and Steinbüchel 2003). ADP1 is a nonmotile coccus with a polysaccharide capsule, which consist of D-glucose and L-rhamnose (Taylor and Juni 1961).

A. baylyi has been referred to as *Acinetobacter* sp. strain ADP1 or *Acinetobacter calcoaceticus* ADP1 due to uncertainties regarding the taxonomy of the species. In 2006, Vaneechoutte et al. solved the taxonomic problem.(Vaneechoutte et al. 2006) ADP1 is a mutant (BD413) of a *Acinetobacter calcoaceticus* strain BD4, which was isolated from soil with meso-2,3-butanediol (Juni and Janik 1969).

ADP1 has been studied due to its suitability as a model organism. It is easily transformable as demonstrated by Juni and Janik (Juni and Janik 1969). The transformation system of ADP1 is more similar to Gram-positive naturally transformable bacteria than Gram-negative. ADP1 takes up chromosomal DNA and plasmid DNA via the same system and as it takes up homologous DNA it can also take up heterologous. The system involves taking up the DNA as single-stranded and requires Ca^{2+} and/or Mg^{2+} and/or Mn^{2+}.(Palmen et al. 1993)

The transformation system is not yet confirmed, but Averhoff and Graf have suggested a system by monitoring the coding of different proteins and the levels of the proteins in different growth phases (Gerischer 2008). The suggested system is presented in Figure 2.

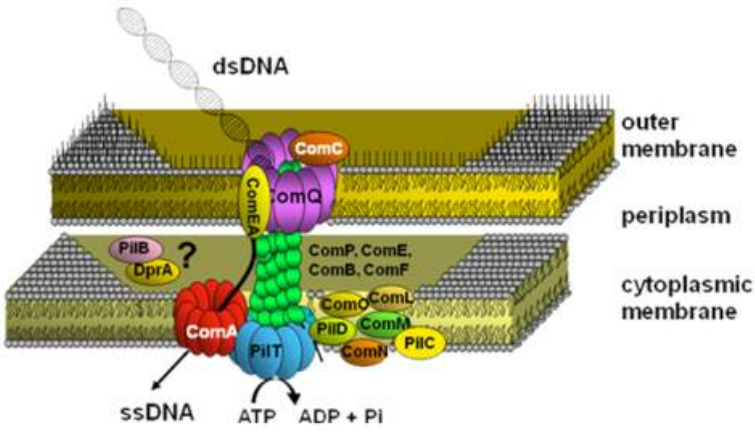


Figure 2. *Suggested DNA uptake in ADP1.(Adapted from Gerischer 2008)*

In this model a protein called ComQ forms a ring in the outer membrane of ADP1. The hole inside the ring is large enough to let DNA pass through the membrane into the periplasm. It is still unclear how the channel would open, but one possible explanation is signals from DNA binding to a protein in the outher membrane. Averhoff and Graf believe that proteins ComB, ComE, ComF and ComP form a pseudopili from the cytoplasmic membrane to the outer membrane. (Gerischer 2008)

Once the DNA reaches the cytoplasmic membrane, it goes through a channel formed by ComA. This mechanisms is assisted by cytoplasmic membrane proteins ComL, ComM, ComN and ComO. Whether energy for the DNA uptake is driven by proton motive force or ATP hydrolysis remains unclear.(Gerischer 2008)

Transformation frequency in ADP1 is the highest when the growth is in exponential phase. After the growth enters the stationary phase, the transformation frequency decreases significantly.(Palmen et al. 1994)

The transformation frequency of integrating heterologous DNA into the genome of ADP1 is drastically lower than that of homologous DNA. However, the frequency is increased when the foreign DNA contains a homologous sequence with ADP1 genome.(de Vries and Wackernagel 2002) In addition, the genome of ADP1 is similar compared to *Escherichia coli* and as genetic information suggest ADP1 is not pathogenic and thus suitable for laboratory use (Metzgar et al. 2004; Barbe et al. 2004).

2.2.1 ADP1 Wax Ester Production

Wax esters (WE) are esters of long-chained acids (i.e. fatty acids) and fatty alcohols (Holum 1990; Schmid 1995). The stoichiometric equation below presents the chemical reaction involved in forming wax esters. (O'Lenick 2007)

$$\mathrm{R{-}\overset{\overset{\mathrm{C}}{\|}}{\mathrm{C}}{-}OH} + \mathrm{R{-}OH} \rightarrow \mathrm{R{-}\overset{\overset{\mathrm{O}}{\|}}{\mathrm{C}}{-}O{-}R} + H_2O,$$

where R is a carbon chain.

WEs are used e.g. in cosmetics, food industry, printing inks and pharmaceuticals. E.g. Cetyl palmitate ($C_{32}H_{64}O_2$) is used in cosmetics.(Schmid 1995) The sources of wax esters include some plants (e.g. jojoba) and fish. They can also be found in beeswax and the surface waxes of insects. (Holum 1990; Ishige et al. 2002)

ADP1 produces WEs from 30 carbons up to 36 carbons. The genetic engineering of ADP1 for storage lipid production has been studied (Santala et al. 2011a; Santala et al. 2014). The suggested wax ester production pathway in ADP1 is presented in Figure 3.

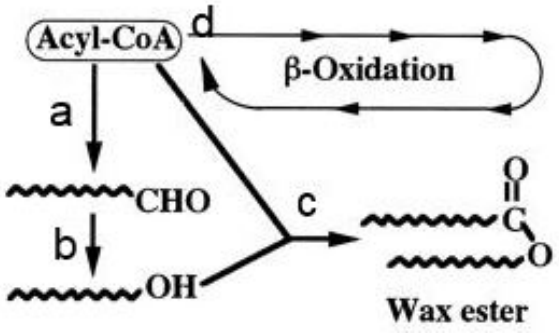


Figure 3. *Suggested wax ester production from acyl-CoA in ADP1. a, acyl-CoA reductase; b, aldehyde reductase; c, wax ester synthase/acyl-CoA:diacylglycerol acyltransferase; d, acyl-CoA dehydrogenase.(Adapted from Ishige et al. 2002)*

The biosynthesis of wax esters in ADP1 begins with the reduction of acyl coenzyme A (acyl-CoA) into a long-chain aldehyde with acyl-CoA reductase. The aldehyde is then reduced into a fatty alcohol with aldehyde reductase.(Kalscheuer and Steinbüchel 2003)

Finally the alcohol is esterified with fatty acyl-CoA into a wax ester. This is catalyzed by wax ester synthase/acyl-CoA:diacylglycerol acyltransferase (WS/DGAT), which is essential to WE accumulation in ADP1. WS/DGAT is bifuctional as it catalyzes reactions to WEs and TAGs.(Kalscheuer and Steinbüchel 2003) WS/DGAT is a highly unspesific enzyme and it can utilize fatty alcohols and acyl-CoAs with different chain lenghts, alkanethiols and e.g. 1-monopalmitoylglycerol (Uthoff et al. 2005).

In the absence of WS/DGAT coding gene, wax/dgat, no WEs are accumulated but small amounts of TAGs can be produced in ADP1. Thus WE production via WS/DGAT is the only route for WE accumulation in ADP1. Similar proteins to WS/DGAT are coded in many *Mycobacterium.*(Kalscheuer and Steinbüchel 2003)

WEs might have a function as an energy reserve molecules in ADP1. In a study by Fixter et al., WEs were degraded when the studied bacteria were subjected to carbon starvation conditions. The highest wax ester content obtained in the study was 141.4 mg*(g dry bacteria weight)$^{-1}$ in nitrogen limited conditions and 56.5 mg*(g dry bacteria weight)$^{-1}$ in carbon limited.(Fixter et al. 1986)

If ADP1 did not consume wax esters as energy reserves, ADP1 might produce WEs in nitrogen-rich conditions as well. This could be achieved by stopping the cell division. When the cells do not need ATP for cell division, WEs could be stored even in nitrogen-rich conditions. The disruption of cell division has been used with other organisms in different studies in order to increase intracellular metabolite yield. The group of Tan achieved 19 % increase in the yield of polyhydroxyalkanoates in *Halomonas* TD01 and Wang et al. were able to get an over 100 % in poly(3-hydroxybutyrate) in *E. coli.*(Tan et al. 2014; Wang et al. 2014)

2.3 A cytoskeleton-binding toxin, cbtA

CbtA is a cytoskeleton-binding toxin found in *E. coli.* It interacts with two different cytoskeleton proteins, FtsZ and MreB in order to prevent cell division. CbtA has been previously called YeeV, but cytoskeleton-binding toxin describes better its function. (Tan et al. 2011)

MreB is a protein, which is thought to be the homologue of actin. MreB forms filaments and controls the shape of bacteria like *E. coli.* Bacteria that have a MreB gene are usually shaped rod-like.(Jones et al. 2001)

FtsZ is an essential protein for cell division and is the homologue of tubulin (Erickson 1995). For a bacterium cell to divide FtsZ must be present. The disruption of coding of the FtsZ gene results in longer cells that do not divide.(Dai and Lutkenhaus 1991) The inhibiton of the function of these two proteins prevents the cells from dividing, which is the working principle of cbtA (Tan et al. 2011).

Cbta has an antitoxin called YeeU, which is coded upstream of cbtA in *E. coli* genome (Brown and Shaw 2003a). Binding of the antitoxin into the toxin is the most common method of inhibiting the function of the toxin (Jensen and Gerdes 1995). However, YeeU does not function in this way. YeeU binds to MreB and FtsZ and stabilizes the proteins.(Masuda et al. 2012)

2.4 Increased Production of Storage Lipids by Metabolic Engineering

Most studies concerning metabolic engineering to increase storage lipid content concentrate on triacylglycerol. TAGs are synthesised in both eukaryotes and prokaryotes. One vastly used prokaryote in TAG studies is *Saccharomyces. cerevisiae*. The biosynthesis of TAGs in *S. cerevisiae* starts from glycerol-3-phosphate (G3P).(Sorger and Daum 2003)

G3P is then converted into lysophosphatidic acid (LPA) with glycerol-3-phosphate acyltransferase (GPAT). LPA can be formed from dihydroxyacetone-phosphate through 1-acyl-DHAP as well. LPA transforms into phosphatidic acid (PA) catalysed by acyl-G3P acyltransferase (AGAT). PA is then converted into DAG via phosphatidate phosphatese (PAP) and finally into TAG with DGAT. (Sorger and Daum 2003) This pathway is presented in Figure 4.

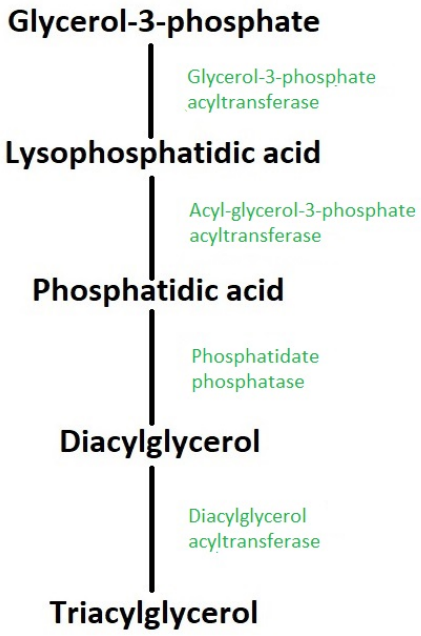


Figure 4. *Triacylglycerol synthesis in Saccharomyces cerevisiae.*

The enzymes in this pathway are often the target of metabolic engineering when improvement of TAG content is the goal.

2.4.1 Yeasts

Different yeasts have genes for diacylglycerol acyltransferases (DGATs). There are two types of DGATs, DGAT1 and DGAT2.(Liu et al. 2012) *S. cerevisiae* can only code DGAT2, which are smaller molecules than DGAT1s (McFie et al. 2010). However it has been shown, that DGAT1 is more important in TAG accumulation at least in plants (Liu et al. 2012). DGAT1s are membrane-bound acyltransferases with a size of approximately 60 kDA (McFie et al. 2010).

Greer et al. were able to increase TAG production in *S. cerevisiae* by introducing DGAT1 coding genes to it. Four different DGAT1s from oil crop *Brassica napus* (BnaDGAT1s) were identified and overexpressed in *S. cerevisiae*. Each of these BnaDGAT1s increased the TAG accumulation in the yeast.(Greer et al. 2015)

They were able to further increase the TAG content by the modification of the N-terminal of the BnaDGAT1. The N-terminal tag increased the DGAT1 content in the cells and thus finally the TAG content. The best strain resulted in 6.4 % TAGs/ dry cell weight, which is a 1.3-fold increase compared to the wild type.(Greer et al. 2015)

Metabolic engineering of *S. cerevisiae* was used to increase storage lipid content already over a decade ago. The group of Karin Athensteadt used different gene deletions to study the affect of these genes on lipid production in *S. cerevisiae*. By deleting the open reading frame for YMR313c, they got a 2.5-fold inrease in TAG content (Athenstaedt et al. 1999). The function of the product of the gene was unknown at time of the study , but it was identified as a TAG lipase later (Athenstaedt and Daum 2003).

Yu et al. used two different approaches to increase the TAG content in *S. cerevisia* using glycerol as a carbon source. First, they overexpressed glycerol kinase (GUT1) to increase glycerol-3-phosphate levels in the cell, which led to 2.4-fold increase in G3P levels. This approach resulted in 1.4-fold increase in TAG accumulation.(Yu et al. 2013)

The second approach was to overexpress LRO1 for phospholipid:diacylglycerol acyltransferase and DGA1 for diacylglycerol acyltransferase. These enzymes are involved in the formation of TAG from diacylglycerol. Both genes were overexpressed individually and together. All three cases showed an increase in TAG content. The best

TAG yield was 8.2 wt% corresponding a 2.3-fold increase with a strain with all three overexpressions.(Yu et al. 2013)

Dulermo and Nicaud increased *Y. lipolyticas* TAG yield by increasing the production of glycerol-3-phosphate in the yeast cells. They experimented with many different *Y. lipolytica* strains with different genetic modifications. In addition to these existing strains a NAD+-dependent G3P dehydrogenase (Gpd1) coding gene GPD1 was overexpressed.(Dulermo and Nicaud 2011)

The highest G3P yield resulting in the highest TAG concentration was a strain with deletion of a mitochondrial FAD+-dependent G3P dehydrogenase coding gene GUT2. With these modifications the G3P yield was 5.6-fold higher than in the wild type. It also yielded in a higher TAG to free fatty acid ratio and in a higher overall lipid content. Without GUT2 inactivation the overexpression of GDP1 did not have a significant affect on G3P levels.(Dulermo and Nicaud 2011)

Qiao et al. identified a delta-9 stearoyl-CoA desaturase (SCD) as a rate limiting step in lipid production of obese mammalian cells. They overexpressed SCD together with acetyl-CoA carboxylase (ACC1) and DGA1 in *Y. lipolytica*. SCD alone did not increase the lipid content in *Y. lipolytica*, but changed the ratio of saturated and unsaturated fatty acids towards unsaturated.(Qiao et al. 2015)

After overexpressing all three genes under a strong constitutive TEF-intron promoter, they were able to increase the lipid accumulation in the yeast. Resulting TAG accumulation was 11.5-fold compared to the wild type, representing 23 wt% TAGs.(Qiao et al. 2015)

2.4.2 Algae

Hsiesh et al. transformed *Chlorella minutissima* with a plasmid containing genes for DGAT, glycerol-3-phosphate acyltransferase (GPAT), glycerol-3-phosphate dehydrogenase (G3PDH), lysophosphatidic acid acyltransferase (LPAAT) and phosphatidic acid phosphatase (PAP). All of the used enzymes have a role in TAG accumulation. The plasmid had homologous sequences with *C. minutissima* genome and two promoters, a ribulose-1,5-bisphosphate carboxylase small subunit (RbcS) and a cauliflower mosaic virus 35s (CaMV).(Hsieh et al. 2012) Schematics of the genetic circuit are in Figure 5.

Figure 5. *The genetic circuit used by the group.(Adapted from Hsieh et al. 2012) DGAT, diacylglycerol acyltransferase; G3PDH, glycerol-3-phosphate dehydrogenase; GPAT, glycerol-3-phosphate acyltransferase;LPAAT, lysophosphatidic acid acyltransferase; PAP, phosphatidic acid phosphatase;* $P_{CaMV35s}$*, cauliflower mosaic virus 35s promoter;* P_{RBCs}*, ribulose-1,5-biphosphate carboxylase small subunit promoter.*

By expressing all of these gene the group achieved 2-fold increase in TAG content compared to the wild type resulting in 44 wt% TAG. Expression of a portion of these genes resulted in an increased TAG levels as well, but the largest yield was with the construct containing all of the used genes.(Hsieh et al. 2012)

Chlamydomonas reinhardtii is a green microalga, which accumulates lipids, including TAGs under nitrogen and/or phosphorous depletion.

The group of Li used a transformed strain of *C. reinhardtii* called BAFJ5 to increase TAG production in the alga (Li et al. 2010). BAFJ5 mutant, transformed previously by Zabawinski et al., lacks genes for ADP-glucose pyrophosphorylase (AGPase) small subunit (Zabawinski et al. 2001). AGPase is a key enzyme in algae starch formation and the starch pathway uses some same carbon precursors than the production of TAG (Li et al. 2010).

The transformant and the wild type were grown both on low light intensity conditions and on high light intensity together with nitrogen starvation. In the low light conditions, no TAGs were present in either of the strains. However, in high light intensity, BAFJ5 produced 10 times more TAGs than the WT. Stopping starch formation in *C. reinhardtii* resulted in 20.5 % of TAGs per dry weight.(Li et al. 2010)

C. reinhardtii has citrate synthases (CIS) in its mitochondria. It catalyses the formation of citroylâCoA from acetyl-CoA, which is the first step in tricarboxylic cycle. Deng et al. experimented wheter the expression of CIS has effect on lipid production in *C. reinhardtii.* They both overexpressed and deleted the gene to asses this.(Deng et al. 2013)

Overexpressing the gene resulted in lower lipid and TAG levels as expected, even though CIS does not directly affect lipid production. On the contrary, supressing the

gene increased TAG accumulation. Finally *C. reinhardtii* was able to accumulate TAGs 1.7-fold compared to the WT.(Deng et al. 2013)

The group of Úbeda-Mínguez tried to increase TAG production in *Tetraselmis chui* with two DGATs. They introduced *T. chui* with DGAT1 from *Echium pitardii* and DGAT2 from *S. cerevisiae* separately. Five different clones had over 1.4-fold increase in TAG content compared to the wild type. The best clone was transformed with DGAT2 and had 2-fold increase in TAG.(Úbeda-Mínguez et al. 2017)

2.4.3 Bacteria

In 2013, Kurosawa et al. engineered *Rhodoccus opacus* strain PD630 to produce TAGs from xylose. Wild type *R. opacus* is capable of producing TAGs but it can not utilize xylose as a carbon source. The group transformed PD630 with genes from xylose utilizing *Streptomyces padanus* MITKK-103. Out of 42 transformants 4 were able to produce TAGs in high xylose concentration. The best strain reached a TAG yield of 12 g/l in 120 g/l xylose.(Kurosawa et al. 2013)

The group of Hernández were able to engineer *Mycobacterium smegmatis* to accumulate TAGs in nitrogen-rich conditions. *M. smegmatis* is a Gram-positive bacteria, which is able to produce TAGs in nitrogen limited conditions. However, an increase in TAG accumulation in nitrogen-rich conditions was achieved by transforming *M. smegmatis* with a WS/DGAT coding gene (atf2) from *R. opacus*. In addition, the transformated *M. smegmatis* had a decreased production of TAGs compared to the wild type in nitrogen limited conditions.(Hernández et al. 2013) The reviewed studies are summarized in Table 1.

Table 1. *Enhanced storage lipid production in microbes achieved by metabolic engineering.*

Species	Product	Produced Lipids (% dry cell weight)	Study
Algae			
C. minutissima	TAG	44	Hsieh et al. 2012
C. reinhardtii	TAG	6.3	Deng et al. 2013
C. reinhardtii	TAG	21	Li et al. 2010
T. chui	TAG	1.3	Úbeda-Mínguez et al. 2017
Yeasts			
S. cerevisiae	TAG	6.4	Greer et al. 2015
S cerevisiae	TAG	8.2	Yu et al. 2013
Y. lipolytica	TAG	35	Dulermo and Nicaud 2011
Y lipolytica	TAG	23	Qiao et al. 2015
Bacteria			
R. opacus	TAG	46	Kurosawa et al. 2013

2.5 Production of Value Compounds from Nitrogen-rich Substrate

Industrial bioprocesses yield protein by-products, which are used as animal feed. However, the amount of n-rich by-products exceed the demand for animal feed and it is important to utilize them otherwise as well.(Wijffels and Barbosa 2010) The processes also result in additional nitrous oxide, which is a strong greenhouse gas (Miller 2010).

Although there are only a few studies where storage lipids are produced from nitrogen rich substrate, the production of other valuable compounds from n-rich substrate have been studied more widely.

E.g. Huo et al. produced isobutanol by *E. coli* from protein rich substrate. This was achieved with a series of genetic modifications including three exogenous nitrogen deamination and transamination cycles. First they added the isobutanol synthesis pathway and overexpressed the key genes for the pathway. This resulted in only 2.3 % theoretical isobutanol yield. Then it was discovered that deleting genes involved in autoinducer-2 (AI-2) synthesis and reuptake increased the yield further. AI-2

reduces cell growth when cells face stress, which is the case in isobutanol production.(Huo et al. 2011)

After this the three transamination and deamination cycles where transformed into the bacteria. They resulted in a irreversible trap for releasing ammonia and formation of puryvate. With this strain they were able to achieve 56 % theoretical yield of alcohols inclunding 50 % isobutanol.(Huo et al. 2011)

3. MATERIALS AND METHODS

The materials and methods used in this work are introduced in this chapter. This includes constructing the plasmid used in this work and its transformation into ADP1 and different growth tests.

3.1 Cultivations

Majority of the cultivations were performed in lysogeny broth (LB) (Bertani 1951). MA/9 medium, containing 0.24 g/l $MgSO_4$, 0.022 g/l $CaCl_2$, 2.0 g/l casein amino acids, 5.5 g/l Na_2HPO_4*$2H_20$, 3.4 g/l KH_2PO_4, 0.94 g/l NH_4Cl, 8.0 mg/l nitrilotriacetic acid, 1.0 g/l NaCl, was used to study the wax ester production in *Acinetobacter baylyi*.

Four different ADP1 strains were cultivated in nitrogen rich media, in order to select the best strain for further studies. Selected strains were ADP1 wild-type, fatty-acyl-Coenzyme A reductase (ACIAD3383/acr1) overexpression strain (S3), isocitrate lyase (ACIAD1084/aceA) deletion strain (S1) and ACIAD1187-90 deletion strain (S2). ACIAD1187-90 genes are for a isocitrate dehydrogenase (idh), a putative transthyretin-like protein, a putative pseudouridine synthase and a isocitrate dehydrogenase (icd), respectively. The strains were pre-cultured in LB agar plates at 30 °C. ACIAD3383 overexpression strain was precultured with 50 μg/ml spectinomycin, ACAD1084 with 50 μg/ml kanamycin and ACIAD1187-90 with 25 μg/ml chloramphenicol. The same pre-culture method and same antibiotics and concentrations were used throughout the study, if not mentioned otherwise. The strains, their abbreviations and the corresponding antibiotics are presented in Table 2.

Table 2. *ADP1 strains used in this study. The antibiotics for each strain were used in concentrations presented in the Table, if not mentioned otherwise.*

Strain	In text	Antibiotic
Wild type	**WT**	-
ACIAD1084	**S1**	50 μg/ml kanamycin
ACIAD1187-90	**S2**	25 μg/ml chloramphenicol
ACIAD3383 overexpression	**S3**	50 μg/ml spectinomycin

3.2 Lipid Extraction

A lipid extraction was performed for samples studied with thin layer chromatography (TLC). 3 ml of a sample was centrifuged for 3 min with 10,000 g. Then the supernatant was removed and 500 μl methanol was added to the sample. The sample was mixed until the bacterial colonies were re-suspended. After adding 250 μl choloform to the sample, 1 h of slow rotation in room temperature was performed. The sample was then centrifuged for 5 min with 10,000 g and 250 μl chloroform and phosphate-buffered saline (pH 7) were added. The sample was kept on slow rotation at 4 °C overnight and centrifuged for 5 min with 10,000 g.

3.3 Thin Layer Chromatography

The samples from lipid extraction were concentrated by evaporating all liquid and adding 50 μl chloroform. A TLC was operated for the samples with n-hexane : diethyl ether : acetic acid 90:15:1 as the mobile phase. Merck TLC Silica gel 60 F_{254} glass plates (20x20 cm) were used. Jojoba oil was used as the standard and iodine for the visualization.

3.4 High Partition Liquid Chromatography

Shimadzu LC-20AD Liquid Chromatograph was used for the high partition liquid chromatography (HPLC) measurements. Shimadzu RID-10A, a refractive index

detector and Phenomenex RHM-Monosaccharide column were used. 0.01 N H_2SO_4 was used as the mobile phase.

The samples for the HPLC measurements were diluted 20-fold and filtered through 0.20 μm filter. A standard curve consisting of concentrations of 0.05, 0.1 and 10 mM acetate was used the determine the acetate concentrations in the samples.

3.5 Selection of Chassis

In order to asses the wax ester production of different ADP1 strains, all four strains were cultivated in nitrogen-rich media containing 10 g/l tryptone and in carbon-rich media containing 10 g/l tryptone and 50 g/l glucose. Strain S3 was cultured both with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and without. Optical densities (OD) of these cultures were measured after 24 h of cultivating in 30 °C and 300 rpm in a shaker. Ultrospec 500 pro spectrophotometer and 600 nm wavelength was used for the OD measurements (OD600).

The same experiment was performed with 20 g/l tryptone and OD600 measurements after 24 h and 48 h. In addittion, ADP1 WT and S1 were cultivated in different glucose concentrations with 20 g/l tryptone. Chosen concentrations were 0 %, 1 %, 2 % and 5 % glucose. The strains were cultivated for 48 h in 30 °C and 300 rpm. After the cultivation OD600 and TLC measurements were performed.

3.6 Stopping the Growth with Acetate

S1 can not utilize acetate as a carbon source. The effect of disrupting the cell division was studied by growing both S1 and WT strains in 5 % glucose and then continuing the growth in 50 mM acetate. The strains were pre-cultured with LA plates and then in MA/9 containing 5 % glucose. 300 μ of pre-culture was added into 20 ml MA/9 with 5 % glucose. The mixture was divided in four 5 ml cultures. The cultures were grown for 24 h at 30 °C and 300 rpm.

After 24 h ODs were measured and TLC samples were taken from two cultures. The supernatants from the TLC samples preparations were kept for HPLC. The two other cultures were centrifuged with 10,000 rpm and the supernatant was removed. The cells were washed with MA/9 and then resuspended with MA/9 containing 50 mM

acetate. 5 ml cultures with MA/9 and acetate were grown for 16 h at 30 °C and 300 rpm. All new cultures contained the same amount of biomass based on the OD600 results.

After the cultivation OD, TLC and HPLC samples were taken. The same procedure was used with both strains. A standard straight of 0.05, 0.1 and 10 mM acetate was used for the HPLC measurements.

3.7 Stopping the Growth with Chloramphenicol

Another approach used for stopping the growth was using chloramphenicol as an antibiotic. ADP1 WT was cultivated in 5 % glucose, 5 % glucose and 10 g/l tryptone, 5 % glucose and 20 g/l tryptone and in 20 g/l tryptone. Six cultivations were grown at 30 °C and 300 rpm for each carbon source variation. After 24 h of cultivating in MA/9 medium without casein amino acids, OD600 was measured and duplicate TLC samples were taken for each variation.

After sampling, 25 μg/ml chloramphenicol was added into two cultivations and 1 μl/ml 70 % ethanol into two cultivations in order to control the effect of ethanol in the chloramphenicol. After a cultivation of 24 h, OD600 and pH were measured from all cultivations and TLC samples were taken from cultivations that contained chloramphenicol or ethanol.

3.8 Construction of Genetic Circuits

The template used for b2005 amplification was the genome of *E. coli* MG1655. The genome isolations were performed with Thermo Scientific GeneJET Genomic DNA Purification Kit and according to its instructions. The DNA concentration in the isolate was measured with Nanodrop 2000 UV-Vis spectrophotometer.

MJ Research PTC-200 DNA Engine was used for the polymerase chain reaction. The step temperatures were 98 °C , 55 °C and 72 °C , respectively. The denaturation, the annealing and the elongation steps were 10 s, 10 s and 30 s, respectively. 35 cycles were run. Phusion Hot Start II (Thermo Fisher Scientific) was used as the DNA polymerase and the buffer used was HF buffer (Finnzymes). The primers used for the amplification are presented in Table 3.

Table 3. *Primers used for b2005 amplification.*

Primer	**Sequence 5' –> 3'**
ab149	TAATACATATGAAAACATTACCTGTATTACCCGGGCAG
ab150	TAATACTCGAGTTATTTCGCCTCCGGATACTTACCCAGG

The PCR products were verified with agarose gel electrophoresis (1 % agarose) and Thermo Scientific GeneJET PCR Purification Kit was used for the purification of the PCR product.

The PCR product and arai/pIX plasmid were digested with restriction enzymes NdeI and XhoI and Thermo Scientific 10X Buffer R by incubating 2 h in 37 °C. The arai/pIX plasmid was constructed previously by Santala et al. (Santala et al. 2011b; Santala et al. 2014). The plasmids are listed in Table 4.

Table 4. *Plasmids used in the study.*

Plasmid	**Study**
pBAV1C-ara-luxCDE	(Santala et al. 2014)
i-luxAB	(Santala et al. 2011b)
araib2005/pIX	This work

The insert and the vector were run in an agarose gel for 2 h at 50 V and the appropiate bands for the ligation were cut from the gel. The DNAs were extracted from the gels with Thermo Scientific GeneJET Gel Extraction Kit. The digestion products were ligated by incubating in room temperature overnight. T4 DNA Ligase was used as the ligase enzyme.

The ligated plasmid was transformed into *E. coli* KRX competent cells. Transformation was conducted by electroporation with VWR electroporation cuvettes (1 mm gap size) and Bio-Rad Micropulser. Immediately after electroporation 1 ml LB-medium was added in the temperature of 37 °C. The cells were let to adapt for 1 h in 37 °C and were plated at LA plates with 25 μg/ml chloramphenicol. The plates were cultured overnight.

The transformation was confirmed by extracting the plasmid with GeneJET Plasmid Miniprep Kit and performing a restriction with same components than earlier. The verification was conducted by agarose gel electrophoresis. The compostion of the plasmid is presented in Figure 6.

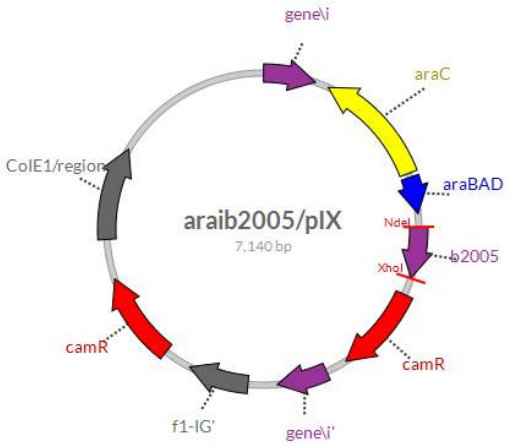


Figure 6. *Araib2005/pIX plasmid constructed in this study.*

The constructed araib2005/pIX plasmid was transformed into *E. coli* Oneshot TOP10 competent cells (Thermo Fisher Scientific 2015b) in order to evaluate the toxicity of the cbtA.

3.9 Transformation

The transformation of ADP1 cells was performed according to Metzgar et al. (2004). The cells were cultivated in LB medium with 1 % glucose over night at 30 °C and 300 rpm. After the cultivation, the growth had reached stationary phase and 50 μl of the stationary culture was added into 2 ml fresh LB. After the cells reached the exponential growth phase, 1 μg insert DNA was added into 0.5 ml culture. The mixture was then cultivated for 2 to 4 h and plated in LB agar with 50μg/ml chloramphenicol. The plates were grown in 30 °C or room temperature until colonies appeared. Controls without insert DNA were cultivated following the same procedure.

3.10 Testing the Effect of cbtA

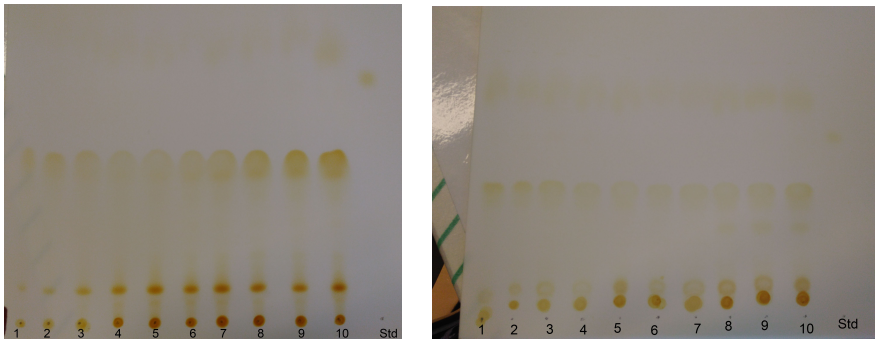
ADP1 WT and S1 transformed with araib2005/pIX were cultivated with 0 %, 0.1 % and 1.0 % arabinose to asses the effect of cbtA to cell division. LB medium with 1 % glucose was used for the cultivations. OD600 was measured from the samples after 2 h, 4h and 24 h of cultivating. ADP1 wild type and S1 were used as controls and *E. coli* TOP10 with the araib2005/pIX plasmid was used to monitor the effect of the cbtA.

4. RESULTS

The results of the conducted studies are presented in this chapter. Thin layer chromatography results are presented with figures of the TLC plates and other results are summarized in tables.

4.1 Selection of Chassis

The optical density results to determine the best strain for nitrogen-rich conditions are presented in Table 5. OD600 was measured for cultivations with 10 g/l tryptone only after 24 h and for cultivations with 20 g/l tryptone after 24 h and 48 h of cultivating. Photos of thin layer chromatography plates for the same cultivations are presented in Figure 7.



(a) (b)

Figure 7. *TLC results for different ADP1 strains: (a) Bacteria grown for 24 h with 10 g/l tryptone. (b) Bacteria grown for 48 h with 20 g/l tryptone. 1, ADP1 WT carbon-rich; 2, ADP1 WT nitrogen-rich; 3, ADP1 S1 carbon-rich; 4, ADP1 S1 nitrogen-rich; 5, ADP1 S2 carbon-rich; 6, ADP1 S2 nitrogen-rich; 7, S3 carbon-rich; 8, ADP1 S3 nitrogen-rich; 9, ADP1 S3 carbon-rich and 1mM IPTG induction; 10, ADP1 S3 nitrogen-rich and 1mM IPTG induction; Std, Jojoba oil.*

Table 5. *Optical densities for different ADP1 strains. IPTG, isopropyl β-D-1-thiogalactopyranoside*

ADP1 strain	Tryptone (g/l)	Glucose (g/l)	Induction	OD 24 h	OD 48 h
Wild type	10	50	-	1.5	-
	10	-	-	1.4	-
	20	50	-	1.1	2.3
	20	-	-	2.4	2.4
ACIAD1084	10	50	-	1.2	-
	10	-	-	1.3	-
	20	50	-	0.9	1.3
	20	-	-	0.9	1.3
ACIAD1187-90	10	50	-	1.2	-
	10	-	-	1.2	-
	20	50	-	1.0	1.6
	20	-	-	2.3	2.5
ACIAD3383	10	50	-	1.0	-
overexpression	10	-	-	1.2	-
	10	50	1 mM IPTG	0.9	-
	10	-	1 mM IPTG	1.1	-
	20	50	-	2.3	2.4
	20	-	-	2.2	1.6
	20	50	1 mM IPTG	1.2	1.7
	20	-	1 mM IPTG	1.0	1.8

There was slight wax ester accumulation in both wild type and both S1 samples with 20 g/l tryptone. The accumulation was so slight that it can not be seen in Figure 7(b). Due to this WE accumulation, the ACIAD1084 deletion strain (S1) was selected for the host for further studies.

4.2 Transformation

The verification of plasmid ligation and transformation into KRX was perfomed by agarose gel electrophoresis. The visualization of the gel with the insert restricted from the plasmid is presented in Figure 8.

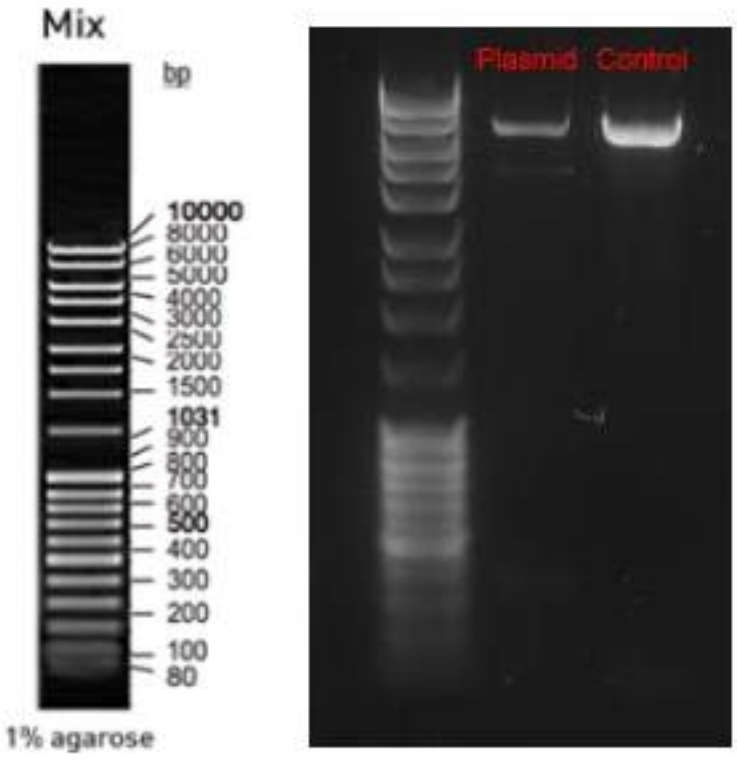


Figure 8. *Plasmid verification by agarose gel electrophoresis. MassRuler DNA Ladder (Thermo Fisher Scientific 2015a) on the left and the result of the gel electrophoresis on the right. There is a faint band corresponding 400 bp in the plasmid sample.*

A slight band of 400 base pairs can be seen in the Figure. The length corresponds the length of the amplificated b2005 insert, which confirms that the ligation of the insert into the plasmid and the transformation of the plasmid into KRX were both successful.

4.3 Arabinose Induction

The OD600 results for araib2005/pIX mutants and their controls with 0, 0.1 and 1 % arabinose are in Table 6. The table includes optical densities after 2, 4 and 24 h of arabinose induction.

Table 6. *OD600 results with different arabinose levels for ADP1 wild type, S1 and araib2005/pIX mutants.*

Strain	Arabinose %	OD 2 h	OD 4 h	OD 24 h
ADP1 WT	**-**	0.95	2.1	9.6
	0.1	0.96	2.0	8.6
	1.0	0.92	1.8	6.4
ADP1 WT +	**-**	0.53	1.5	7.9
araib2005/pIX	**0.1**	0.51	1.5	8.2
	1.0	0.49	1.6	6.5
ADP1 S1	**-**	0.31	0.51	3.1
	0.1	0.21	0.20	0.43
	1.0	0.28	0.68	3.4
ADP1 S1 +	**-**	0.40	0.73	1.5
araib2005/pIX	**0.1**	0.39	0.72	1.4
	1.0	0.37	0.72	1.6
***E. coli* TOP10 +**	**-**	0.54	1.4	1.9
araib2005/pIX	**0.1**	0.52	1.4	1.7
	1.0	0.51	1.2	2.0

Arabinose induction should stop or at least slow down cell growth, but it had no effect on it. Thus, further studies could not be performed to estimated whether stopping the cell growth with cbtA would increase WE accumulation in ADP1.

4.4 Stopping the Growth with Acetate

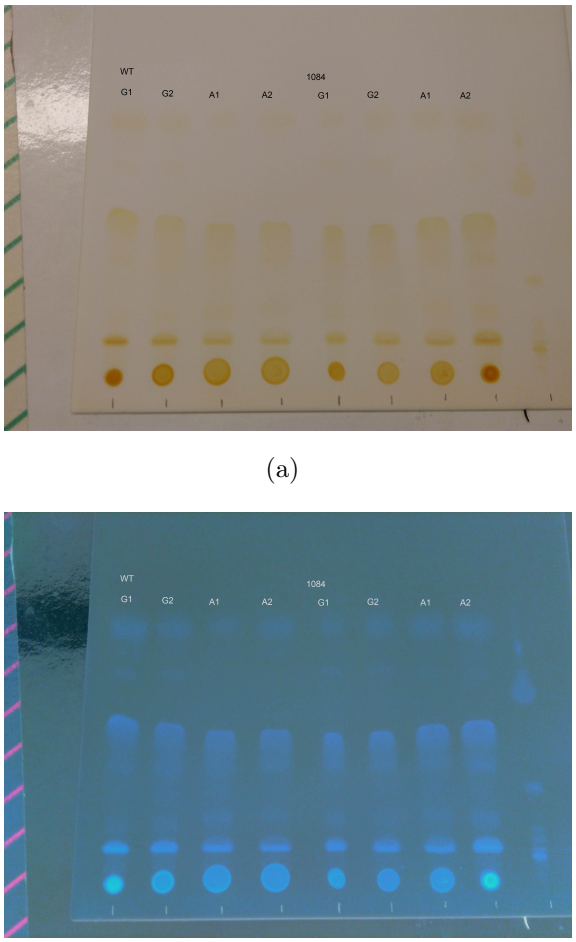
The results for stopping ADP1 ACIAD1084 growth by changing the carbon source from glucose to acetate are discussed in this section. In Table 7, the OD600 results for this experiment are introduced.

Table 7. *Optical densities for cultures grown in 5% glucose for 24 h and afterwards in 50 mM acetate for 16 h.*

Sample	Cultivation Time	Carbon Source	OD
WT	**24 h**	**5 % Glucose**	
1			3.4
2			3.5
3			3.1
4			3.1
WT	**16 h**	**50 mM Acetate**	
3			2.8
4			3.1
S1	**24 h**	**5 % Glucose**	
1			3.1
2			3.2
3			3.1
4			3.9
S1	**16 h**	**50 mM Acetate**	
3			2.0
4			2.0

The table shows that S1 grows approximately as well as WT in glucose. The OD600 of both strains is lower after growth with acetate. This is because the secondary cultures were slightly diluted and same amount of biomass based on the initial OD results was used for all cultures. It can be seen that S1 does not grow on acetate whereas WT does. Since S1 can not utilize acetate as a carbon source these results were as expected. The TLC results for assessing the effect of this stoppage in growth are in Figure 9.

There was no acetate detected in the WT cultures grown with acetate after 16 hours. The two S1 cultures contained 11.4 and 14.0 mM, which are low values compared to the initial 50 mM. Even though the values are low, it can be said that the S1 cultures did not utilize acetate as well as the wild type cultures, which explains the difference in their growth.



(a)

(b)

Figure 9. *TLC results: (a) The original TLC photo. (b) An enhanced photo for better visualization. A1-2, grown on acetate; G1-2, grown on glucose. The rightmost sample is jojoba oil standard.*

There was only slight accumulation of WEs in any of the strains, which makes it difficult to analyse the TLC results. In Figure 9(b), photo manipulation has been used for better visualization of WE accumulation.

Both strains accumulated WEs when grown with 5 % glucose for 24 h. This is consistent with the previous results. S1 accumulated a minimal amount of WEs with acetate as well, but WT did not.

4.5 Stopping the Growth with Chloramphenicol

The results for stopping the growth of ADP1 wild type cultures with differenet carbon sources by chloramphenicol addition are discussed here. The measured optical densities for each culture before and after chloramphenicol addition are presented in Table 8.

Table 8. *Optical densities for ADP1 WT with chloramphenicol addition. Measurement point "before" is before the addition of chloramphenicol or ethanol and "after" is after cultivating 24 h with the additions.*

Carbon Source	Measurement	No Additions	Chloramphenicol	Ethanol
5 % glucose	Before	1.1	0.70	0.63
	After		0.85	3.8
5 % glucose	Before	3.8	3.9	3.9
10 g/l tryptone	After		3.9	3.3
5 % glucose	Before	3.7	3.9	3.5
20 g/l tryptone	After		4.3	3.8
20 g/l tryptone	Before	2.3	2.2	2.2
	After		1.8	2.5

The effect of chloramphenicol is most notable in the cultures with only 5 % glucose as a carbon source. There is a significant difference between the culture with chloramphenicol and the culture with ethanol. In another cultures the addition of chloramphenicol seems to have stopped the growth as well, but in cultures with both glucose and tryptone the culture with added ethanol grew even more poorly than the cultures with chloramphenicol. This should not be the case. The TLC results for this experiment are presented in Figure 10.

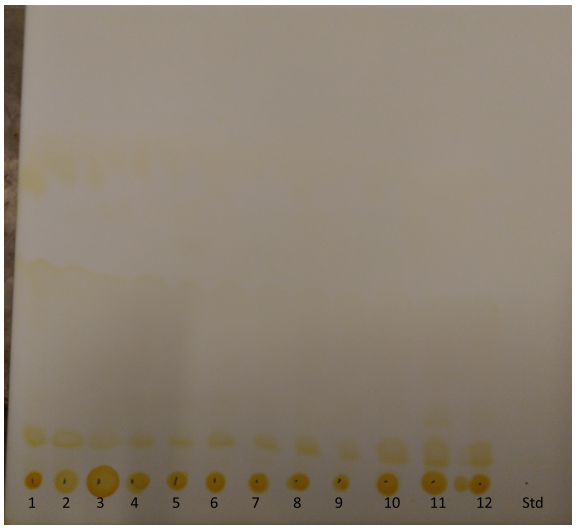


Figure 10. *TLC results for ADP1 WT grown with chloramphenicol addition.* ***1****, 5 % glucose;* ***2****, 5 % glucose, chloramphenicol;* ***3****, 5 % glucose, ethanol;* ***4****, 5 % glucose, 10 g/l tryptone;* ***5****, 5 % glucose, 10 g/l tryptone, chloramphenicol;* ***6****, 5 % glucose, 10 g/l tryptone, ethanol;* ***7****, 5 % glucose, 20 g/l tryptone;* ***8****, 5 % glucose, 20 g/l tryptone, chloramphenicol;* ***9****, 5 % glucose, 10 g/l tryptone, ethanol;* ***10****, 20 g/l tryptone;* ***11****, 20 g/l tryptone, chloramphenicol;* ***12*** *20 g/l tryptone, ethanol;* ***Std****, Jojoba oil.*

Again the WE accumulation was very minimal and the TLC results were difficult to interpret. Figure 10 does not offer any evidence of WE accumulation, but there were slight wax ester bands on the plates. The strongest wax ester bands were in the samples from chloramphenicol culture with 5 % glucose and 10 g/l tryptone and only 20 g/l tryptone. This was confirmed by observing the plate in ultraviolet (UV) light. The UV light showed light bands in other chloramphenicol cultures as well.

5. DISCUSSION

The fact that ADP1 strains transformed with araib2005 genome integration cassette construct did not react to added arabinose, made it impossible to analyse whether cbtA could increase the wax ester content in the ADP1 cells in nitrogen-rich conditions.

In earlier studies, success in expressing the cbtA have been achieved using different bacterial strains, with a low copy number plasmid and with a cultivation medium without glucose (Tan et al. 2011; Brown and Shaw 2003b). These details such as gene copy number might have an effect on the expression and be the reason for unchanged growth in the mutants after arabinose addition.

Even though the disruption of cell growth with cbtA could not be analysed, other cell growth stopping methods and their results can be discussed. The results of the acetate experiment suggest that the hypothesis might be right. WT continued growing on acetate and therefore did not accumulate wax esters whereas S1, which can not utilize acetate as a carbon source, did not grow and was able the preserve some of its WE storage. However, this experiment was done in carbon optimal conditions. Thus, it can not be said that stopping the growth of ADP1 would increase the WE accumulation in nitrogen-rich conditions. The results still support the theory of using WEs as energy reserve.

In the other experiment, the cells with added chloramphenicol had stronger wax ester accumulation than the cells without in nitrogen-rich conditions. This suggests that stopping the growth yields a better WE accumulation as the acetate test suggested as well. In contrast to the acetate experiment, in this experiment the result was achieved with nitrogen-rich cultures as well. Thus, the results support the hypothesis that stopping the cell growth would increase WE levels in nitrogen-rich conditions.

These tests do not answer the question whether higher wax ester content could be achieved by metabolically engineered growth stopping methods in nitrogen-rich

conditions. However with these results, the hypothesis seems possible. The amount of wax esters in these test was not measured, but the levels were quite low. This means that even though it could be possible the increase the WE content by stopping the growth of the cell, much more research would be required to achieve feasible levels.

Utilizing excess nitrogen for storage lipid production by ADP1 might never be feasible. However, with enough research some microbe species might be able to produce valuable compounds out of nitrogen-rich substrate with e.g. metabolically engineered growth stopping methods combined with other modifications.

6. CONCLUSIONS

The araib2005 genome integration cassette construct including the b2005 for coding cbtA and an arabinose inducible promoter did not stop cell division with arabinose induction. Thus, the effect of cytoskeleton-binding toxin on ADP1 cell division and furthermore on wax ester accumulation in nitrogen-rich conditions could not be estimated.

However, the additional experiments concerning the disruption of ADP1 cell division showed promising results. Stopping S1 culture growth by switching the carbon source from glucose to acetate and stopping ADP1 wild type culture growth by introducing chloramphenicol to the cells showed both an increase in wax ester accumulation. In the case of S1, nitrogen limited conditions were used, but the results support the nitrogen-rich case as well. The chloramphenicol test was performed in both conditions and the most notable difference in WE accumulation was found in the nitrogen-rich cases.

As the TLC figures presented in the work show, the wax ester content estimations are highly open to interpretations. All in all it might be possible to use this kind of approach to increase ADP1 wax ester production from nitrogen-rich substrate. If this was to be achieved, a lot of research would have to be done and different cell division disrupting methods had to be used. This study was performed using small scale cultivations, thus there would have to be much more additional researching done with the upscaling as well.

REFERENCES

Athenstaedt, K. and Daum, G. (2003). "YMR313c/TGL3 Encodes a Novel Triacylglycerol Lipase Located in Lipid Particles of Saccharomyces cerevisiae". *The Journal of Biological Chemistry* 278, pp. 23317–23323.

Athenstaedt, K., Zweytick, D., Jandrositz, A., Kohlwein, S. D. and Daum, G. (1999). "Identification and Characterization of Major Lipid Particle Proteins of the Yeast Saccharomyces cerevisiae". *Journal of Bacteriology* 181, pp. 6441–6448.

Bailey, J. E. (1991). "Toward a science of metabolic engineering". *Science* 252, pp. 1668–1675.

Barbe, V., Vallenet, D., Fonknechten, N., Kreimeyer, A., Oztas, S., Labarre, L., Cruveiller, S., Robert, C., Duprat, S., Wincker, P., L. Nicholas Ornston and, J. W., Marliére, P., Cohen, G. N. and Médigue, C. (2004). "Unique features revealed by the genome sequence of Acinetobacter sp. ADP1, a versatile and naturally transformation competent bacterium". *Nucleic Acids Research* 32, pp. 5766–5779.

Bertani, G. (1951). "STUDIES ON LYSOGENESIS I. The Mode of Phage Liberation by Lysogenic Escherichia coli". *Journal of Bacteriology* 62, pp. 293–300.

Brown, J. and Shaw, K. (2003a). "A Novel Family of Escherichia coli Toxin-Antitoxin Gene Pairs". *Journal of Bacteriology* 185, pp. 6600–6608.

– (2003b). "A Novel Family of Escherichia coli Toxin-Antitoxin Gene Pairs". *Journal of Bacteriology* 185, pp. 6600–6608.

Cameron, D. C. and Tong, I.-T. (1993). "Cellular and metabolic engineering". *Applied Biochemistry and Biotechnology* 38, pp. 105–140.

Carr, E. L., Kämpfer, P., Patel, B. K. C., Gürtler, V. and Seviour, R. J. (2003). "Seven novel species of Acinetobacter isolated from activated sludge". *International Journal of Systematic and Evolutionary Microbiology* 53, pp. 953–963.

Cho, K. M., Yoo, Y. J. and Kang, H. S. (1999). "δ-Integration of endo/exo-glucanase and β-glucosidase genes into the yeast chromosomes for direct conversion of cellulose to ethanol". *Enzyme and Microbial Technology* 25, pp. 23 –30.

Dai, K. and Lutkenhaus, J. (1991). "FtsZ is an essential cell division gene in Escherichia coli". *Journal of Bacteriology* 1734, pp. 3500 –3506.

Deng, X., Cai, J. and Fei, X. (2013). "Effect of the expression and knockdown of citrate synthase gene on carbon flux during triacylglycerol biosynthesis by green algae Chlamydomonas reinhardtii". *BMC Biochemistry* 14, p. 38.

Dulermo, T. and Nicaud, J.-M. (2011). "Involvement of the G3P shuttle and β-oxidation pathway in the control of TAG synthesis and lipid accumulation in Yarrowia lipolytica". *Metabolic Engineering* 13, pp. 482 –491.

Erickson, H. P. (1995). "FtsZ, a prokaryotic homolog of tubulin?" *Cell* 80, pp. 367 –370.

Fell, D. A. and Sauro, H. M. (1985). "Metabolic control and its analysis". *European Journal of Biochemistry* 148, pp. 555–561.

Fixter, L. M., Nagi, M. N., McCormacks, J. G. . and Fewson, C. A. (1986). "Structure, Distribution and Function of Wax Esters in Acinetobacter calcoaceticus". *Journal of General Microbiology* 132, pp. 3147–3157.

Gerischer, U. (2008). *Acinetobacter Molecular Biology*. 244 p. Norfolk, UK: Caister Academic Press.

Glick, B. R., Pasternak, J. J. and Patten, C. L. (2010). *Molecular Biotechnology : Principles and Applications of Recombinant DNA*. 1020 p. Washington: ASM Press.

Gombert, A. K. and Nielsen, J. (2000). "Mathematical modelling of metabolism". *Current Opinion in Biotechnology* 11, pp. 180 –186.

Greer, M. S., Truksa, M., Deng, W., Lung, S.-C., Chen, G. and Weselake, R. J. (2015). "Engineering increased triacylglycerol accumulation in Saccharomyces cerevisiae using a modified type 1 plant diacylglycerol acyltransferase". *Applied Microbiology and Biotechnology* 5, pp. 2243–2253.

Hernández, M. A., Arabolaza, A., Rodríguez, E., Gramajo, H. and Alvarez, H. M. (2013). "The atf2 gene is involved in triacylglycerol biosynthesis and accumulation in the oleaginous Rhodococcus opacus PD630". *Applied Microbiology and Biotechnology* 97, pp. 2119–2130.

Holum, J. R. (1990). *Fundamentals of general, organic, and biological chemistry 4th ed.* 792 p. New York: Wiley.

Hsieh, H.-J., Su, C.-H. and Chien, L.-J. (2012). "Accumulation of lipid production in Chlorella minutissima by triacylglycerol biosynthesis-related genes cloned from Saccharomyces cerevisiae and Yarrowia lipolytica". *Journal of Microbiology* 50, pp. 526–534.

Huo, Y.-X., Cho, K., Lafontaine Riviera, J., Monte, E., Shen, C. R., Yan, Y. and Liao, J. C. (2011). "Conversion of proteins into biofuels by engineering nitrogen flux". *Nature* 29, pp. 346–352.

Ishige, T., Tani, A., Takabe, K., Kawasaki, K., Sakai, Y. and Kato, N. (2002). "Wax Ester Production from n-Alkanes by Acinetobacter sp. Strain M-1: Ultrastructure

of Cellular Inclusions and Role of Acyl Coenzyme A Reductase". *Applied and Environmental Microbiology* 68, pp. 1192–1195.

Jensen, R. B. and Gerdes, K. (1995). "Programmed cell death in bacteria: proteic plasmid stabilization systems". *Molecular Microbiology* 17, pp. 205–210.

Jones, L. J., Carballido-Lopez, R. and Errington, J. (2001). "Control of Cell Shape in Bacteria: Helical, Actin-like Filaments in Bacillus subtilis". *Cell* 104, pp. 913 –922.

Juni, E. and Janik, A. (1969). "Transformation of Acinetobacter calco-aceticus (Bacterium anitratum)". *Journal of Bacteriology* 98, pp. 281 –288.

Kalscheuer, R. and Steinbüchel, A. (2003). "A Novel Bifunctional Wax Ester Synthase/Acyl-CoA:Diacylglycerol Acyltransferase Mediates Wax Ester and Triacylglycerol Biosynthesis in Acinetobacter calcoaceticus ADP1". *Journal of Biological Chemistry* 278, pp. 8075–8082.

Ko, J. K., Um, Y., Woo, H. M., Kim, K. H. and Lee, S.-M. (2016). "Ethanol production from lignocellulosic hydrolysates using engineered Saccharomyces cerevisiae harboring xylose isomerase-based pathway". *Bioresource Technology* 209, pp. 290 –296.

Kurosawa, K., Wewetzer, S. J. and Sinskey, A. J. (2013). "Engineering xylose metabolism in triacylglycerol-producing Rhodococcus opacusfor lignocellulosic fuel production". *Biotechnology for Biofuels* 6, p. 134.

Li, Y., Han, D., Hu, G., Dauvillee, D., Sommerfeld, M., Ball, S. and Hu, Q. (2010). "Chlamydomonas starchless mutant defective in ADP-glucose pyrophosphorylase hyper-accumulates triacylglycerol". *Metabolic Engineering* 12, pp. 387 –391.

Liu, Q., Siloto, R. M., Lehner, R., Stone, S. J. and Weselake, R. J. (2012). "Acyl-CoA:diacylglycerol acyltransferase: Molecular biology, biochemistry and biotechnology". *Progress in Lipid Research* 51, pp. 350 –377.

Marx, A., de Graaf, A. A., Wiechert, W., Eggeling, L. and Sahm, H. (1996). "Determination of the fluxes in the central metabolism of Corynebacterium glutamicum by nuclear magnetic resonance spectroscopy combined with metabolite balancing". *Biotechnology and Bioengineering* 49, pp. 111–129.

Masuda, H., Tan, Q., Awano, N., Wu, K.-P. and Inouye, M. (2012). "YeeU enhances the bundling of cytoskeletal polymers of MreB and FtsZ, antagonizing the CbtA (YeeV) toxicity in Escherichia coli". *Molecular Microbiology* 84, pp. 979–989.

Mavrovouniotis, M. (1993). *Identification of qualitatively feasible metabolic pathways*. in: L. Hunter (ed.), *Artificial Intelligence and Molecular Biology*, Springer, New York, USA, pp. 325-364.

McFie, P. J., Stone, S. L., Banman, S. L. and Stone, S. J. (2010). "Topological Orientation of Acyl-CoA:Diacylglycerol Acyltransferase-1 (DGAT1) and Identification of a Putative Active Site Histidine and the Role of the N Terminus in Dimer/Tetramer Formation". *The Journal of Biological Chemistry* 285, pp. 37377–37387.

Metzgar, D., Bacher, J. M., Pezo, V., Reader, J., Döring, V., Schimmel, P., Marliére, P. and Crécy-Lagard, V. de (2004). "Acinetobacter sp. ADP1: an ideal model organism for genetic analysis and genome engineering". *Nucleic Acids Research* 32, pp. 5780–5790.

Miller, S. A. (2010). "Minimizing Land Use and Nitrogen Intensity of Bioenergy". *Environmental Science & Technology* 44, pp. 3932–3939.

Nielsen, J. (1997). "Metabolic engineering: Techniques for Analysis of Targets for Genetic Manipulations". *Biotechnology and Bioengineering* 58, pp. 125–132.

– (2001). "Metabolic engineering". *Applied Microbiology and Biotechnology* 55, pp. 263–283.

O'Lenick, A. J. (2007). *Oils of Nature*. 117 p. Carol Stream, US: Allured Books.

Palmen, R., Vosman, B., Buijsman, P., Breek, C. K. D. and Hellingwerf, K. J. (1993). "Physiological characterization of natural transformation in *Acinetobacter calcoaceticus*". *Journal of General Microbiology* 139, pp. 295–305.

Palmen, R., Buijsman, P. and Hellingwerf, K. J. (1994). "Physiological regulation of competence induction for natural transformation in Acinetobacter calcoaceticus". *Archives of Microbiology* 162, pp. 344–351.

Qiao, K., Abidi, S. H. I., Liu, H., Zhang, H., Chakraborty, S., Watson, N., Ajikumar, P. K. and Stephanopoulos, G. (2015). "Engineering lipid overproduction in the oleaginous yeast Yarrowia lipolytica". *Metabolic Engineering* 29, pp. 56 –65.

Rizzi, M., Baltes, M., Theobald, U. and Reuss, M. (1997). "In vivo analysis of metabolic dynamics in Saccharomyces cerevisiae: II. Mathematical model". *Biotechnology and Bioengineering* 55, 592â608.

Santala, S., Efimova, E., Kivinen, V., Larjo, A., Aho, T., Karp, M. and Santala, V. (2011a). "Improved Triacylglycerol Production in Acinetobacter baylyi ADP1 by Metabolic Engineering". *Microbial Cell Factories* 10, pp. 1–10.

Santala, S., Efimova, E., Karp, M. and Santala, V. (2011b). "Real-Time monitoring of intracellular wax ester metabolism". *Microbial Cell Factories* 10, pp. 1–8.

Santala, S., Efimova, E., Koskinen, P., Karp, M. T. and Santala, V. (2014). "Rewiring the Wax Ester Production Pathway of Acinetobacter baylyi ADP1". *ACS Synthetic Biology* 3, pp. 145–151.

Schmid, G. H. (1995). *Organic chemistry*. 1208 p. St. Louis: Mosby.

Sharan, S. K., Thomason, L. C., Kuznetsov, S. G. and Court, D. L. (2009). "Recombineering: a homologous recombination-based method of genetic engineering". *Nature Protocols* 4, 206â223.

Sorger, D. and Daum, G. (2003). "Triacylglycerol biosynthesis in yeast". *Applied Microbiology and Biotechnology* 61, pp. 289–299.

Tan, D., Wu, Q., Chen, J.-C. and Chen, G.-Q. (2014). "Engineering Halomonas {TD01} for the low-cost production of polyhydroxyalkanoates". *Metabolic Engineering* 26, pp. 34 –47.

Tan, Q, Awano, N and Inouye, M (2011). "YeeV is an Escherichia coli Toxin that Inhibits Cell Division by Targeting the Cytoskeleton Proteins, FtsZ and MreB". *Molecular Microbiology* 79, pp. 109–118.

Taylor, W. H. and Juni, E. (1961). "PATHWAYS FOR BIOSYNTHESIS OF A BACTERIAL CAPSULAR POLYSACCHARIDE I.: Characterization of the Organism and Polysaccharide". *Journal of Bacteriology* 81, pp. 688–693.

Thermo Fisher Scientific (2015a). *MassRuler^{TM} DNA Ladders*. Available (accessed on 16.9.2016): https://www.thermofisher.com/fi/en/home/brands/thermo-scientific/molecular-biology/thermo-scientific-nucleic-acid-electrophoresis-purification/dna-electrophoresis-thermo-scientific/dna-ladders-thermo-scientific/massruler-dna-ladders.html.

– (2015b). *One Shot TOP10 Chemically Competent E. coli*. Available (accessed on 21.9.2016): https://www.thermofisher.com/order/catalog/ product/C404010.

Úbeda-Mínguez, P., García-Maroto, F. and Alonso, D. L. (2017). "Heterologous expression of DGAT genes in the marine microalga Tetraselmis chui leads to an increase in TAG content". *Journal of Applied Phycology* 29, pp. 1913–1926.

Uthoff, S., Stöveken, T., Weber, N., Vosmann, K., Erika Klein and, R. K. and Steinbüchel, A. (2005). "Thio Wax Ester Biosynthesis Utilizing the Unspecific Bifunctional Wax Ester Synthase/Acyl Coenzyme A:Diacylglycerol Acyltransferase of Acinetobacter sp. Strain ADP1". *Applied and Environmental Microbiology* 71, pp. 790 –796.

Vallino, J. and Stephanopoulos, G. (1990). *Flux determination in cellular bioreaction networks: applications to lysine fermentations*. in: S.K. Sikdar, M. Bier and P. Todd (ed.), *Frontiers in bioprocessing*, CRC Press, Boca Raton, USA, pp. 205-219.

Vaneechoutte, M., Young, D. M., Ornston, L. N., Thierry De Baere and, A. N., Reijden, T. V. D., Carr, E., Tjernberg, I. and Dijkshoorn, L. (2006). "Naturally Trans-

formable Acinetobacter sp. Strain ADP1 Belongs to the Newly Described Species Acinetobacter baylyi". *Applied and Environmental Microbiology* 72, pp. 932–936.

Varma, A. and Palsson, B. O. (1994). "Metabolic Flux Balancing: Basic Concepts, Scientific and Practical Use". *Bio/Technology* 12, pp. 994–998.

de Vries, J. and Wackernagel, W. (2002). "Integration of foreign DNA during natural transformation of *Acinetobacter* sp. by homology-facilitated illegitimate recombination". *Proceedings of the National Academy of Sciences of the United States of America* 99, pp. 2094–2099.

Wang, Y., Wu, H., Jiang, X. and Chen, G.-Q. (2014). "Engineering Escherichia coli for enhanced production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) in larger cellular space". *Metabolic Engineering* 25, pp. 183 –193.

Wijffels, R. H. and Barbosa, M. J. (2010). "An Outlook on Microalgal Biofuels". *Science* 329, pp. 796–799.

Yu, K. O., Jung, J., Ramzi, A. B., Choe, S. H., Kim, S. W., Park, C. and Han, S. O. (2013). "Development of a Saccharomyces cerevisiae strain for increasing the accumulation of triacylglycerol as a microbial oil feedstock for biodiesel production using glycerol as a substrate". *Biotechnology and Bioengineering* 110, pp. 343–347.

Zabawinski, C., Koornhuyse, N. V. D., Christophe D

Hulst and, R. S., Giersch, C., Delrue, B., Lacroix, J.-M., Preiss, J., and Ball, S. (2001). "Starchless Mutants of Chlamydomonas reinhardtii Lack the Small Subunit of a Heterotetrameric ADP-Glucose Pyrophosphorylase". *Journal of Bacteriology* 183, pp. 1069–1077.